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Fasciation induction by the phytopathogen *Rhodococcus fascians* depends upon a linear plasmid encoding a cytokinin synthase gene

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Rhodococcus fascians **is a nocardiform bacteria that induces leafy galls (fasciation) on dicotyledonous and several monocotyledonous plants. The wild-type strain D188 contained a conjugative, 200 kb linear extrachromosomal element, pFiD188. Linear plasmid-cured strains were avirulent and reintroduction of this linear element restored virulence. Pulsed field electrophoresis indicated that the chromosome might also be a linear molecule of 4 megabases. Three loci involved in phytopathogenicity have been identified by insertion mutagenesis of this Fi plasmid. Inactivation of the *fas* locus resulted in avirulent strains, whereas insertions in the two other loci affected the degree of virulence, yielding attenuated (*att*) and hypervirulent (*hyp*) bacteria. One of the genes within the *fas* locus encoded an isopentenyltranferase (IPT) with low homology to analogous proteins from Gram-negative phytopathogenic bacteria. IPT activity was detected after expression of this protein in *Escherichia coli* cells. In *R.fascians*, *ipt* expression could only be detected in bacteria induced with extracts from fasciated tissue. *R.fascians* strains without the linear plasmid but containing this *fas* locus alone could not provoke any phenotype on plants, indicating additional genes from the linear plasmid were also essential for virulence. These studies, the first genetic analysis of the interaction of a Gram-positive bacterium with plants, suggest that a novel mechanism for plant tumour induction has evolved in *R.fascians* independently from the other branches of the eubacteria.**

Key words: fasciation/isopentenyltransferase/isopentenyltransferase gene induction/linear plasmid/*Rhodococcus fascians*

Introduction

Few interactions between plants and bacteria lead to the alteration of the normal developmental program of the host partner. Each of the hyperplasia-inducing bacteria, *Agrobacterium tumefaciens* and *Pseudomonas syringae* pv. *savastanoi*, contain genes that synthesize primary plant growth hormones (auxin and cytokinin) (Morris, 1986) and genes that derivatize them (Roberto and Kosuge, 1987). In the first case, the T-DNA carrying these genes induces uncontrolled proliferation of the tissue after transfer to plants (Zambryski, 1988), whereas *Pseudomonas* spp. secrete large amounts of hormones (and several chemical derivatives of them) which are apparently responsible for disease induction (Roberto and Kosuge, 1987). On the other hand, *Rhizobium* spp. and *Agrobacterium rhizogenes*, form new organs (nodules or roots, respectively) as a consequence of bacterial infection. The former bacteria synthesize an extracellular symbiotic signal (the oligosaccharin NodRm-1; Lerouge *et al.*, 1990) that triggers root hair deformation and finally nodule formation on leguminous plants (Long, 1989). The T-DNA of *A.rhizogenes* contains hormone-sensitizing genes (*rol* genes; White *et al.*, 1985), which enhance the effects of auxin and stimulate differentiation of the transformed plant tissue into roots (Shen *et al.*, 1988).

Rhodococcus fascians is a nocardiform (Gram-positive) phytopathogenic bacterial species (LeChevalier, 1986) evolutionarily distinct from the better known Gram-negative bacteria. Infection of dicotyledonous plants by *R.fascians* causes fasciation, a disease characterized by loss of apical dominance and the development of multiple shoots giving rise to leafy galls (Brown, 1927; Tilford, 1936; Stapp, 1961). Upon infection of monocotyledonous plants, such as members of the *Liliaceae*, cauliflower-like structures develop on the bulbs due to malformation of the scubs (Miller *et al.*, 1980; Vantomme *et al.*, 1982). Until now, the molecular basis of this disease has not been elucidated. Early experiments on fasciation indicated that symptoms in sweet peas could be mimicked by the constant application of elevated levels of cytokinins such as kinetin (6-furfuryl amino purine) (Thimann and Sachs, 1966; Roussaux, 1966). Several reports have suggested that secretion of cytokinins by the bacteria is responsible for tumour formation as different cytokinins have been found in the supernatants of several *R.fascians* strains (Klämbt *et al.*, 1966; Scarbrough *et al.*, 1979; Murai *et al.*, 1980).

The location of genes essential for fasciation has been a controversial issue. Lawson *et al.* (1982) found plasmids of ~115 kb both in virulent and in avirulent strains. However, the loss of a 100 kb plasmid from *R.fascians* MW2 was associated with the simultaneous loss of cadmium resistance (Cd^R), cytokinin production and fasciation-inducing properties (Murai *et al.*, 1980). We were able to corroborate this result partially through the characterization of a 138 kb plasmid (pD188) that encodes the cadmium resistance trait of the highly virulent strain D188 (Desomer *et al.*, 1988).

In this paper, we demonstrate that essential fasciation genes in strain D188 are not located on this circular element, but rather on a large conjugative linear plasmid (200 kb) pFiD188 (Fi for 'fasciation inducing'). Moreover, we have identified on this extrachromosomal element three loci involved in phytopathogenicity using an insertion mutagenesis method based on the illegitimate recombination of non-replicating constructs into different positions of the *R.fascians* genome (Desomer *et al.*, 1991). One of these loci (*fas*) codes for an isopentenyl transferase (*ipt*) gene. Bacteria containing only a DNA region of the linear plasmid spanning this *fas* locus could not elicit any phenotype on plants. No significant difference was found in the cytokinin secretion profiles of the wild type and *fas* mutant strains when they

are grown axenically. On the other hand, this *fas* gene is highly induced in the presence of leafy gall extracts. Our results show *R.fascians* has acquired several evolutionary novel features that distinguish it from other bacterial pathogens.

Results

Identification of a fasciation-specific DNA region

To correlate the presence of extrachromosomal elements with the capacity of the harbouring strains to induce fasciation on host plants, we tested the virulence of genetically distinct derivatives of D188 including different pD188-cured strains (Table I). Inoculation of wild-type D188 and the rifampin-cured D188-2 strains, on decapitated axenically grown tobacco plants resulted in the development of typical leafy gall structures with numerous, thickened, dark-green shoots. When the same strains were used to inoculate tobacco seeds, the seedlings remained stunted with a thickened hypocotyl and inhibited leaf development. Cocultivation of leaf discs with these strains resulted in wart-like, green outgrowths (Figure 1). In contrast, none of these phenotypes were observed using the heat-cured D188-1 or the same strain in which pD188 was reintroduced by conjugation (D188-7). These results (Table I) indicate that fasciation functions do not reside on pD188. Consequently, one or more essential fasciation genes must be located on another replicon or chromosome region present in the virulent D188-2 and D188 strains yet absent in D188-1. Therefore, we screened a D188 genomic library with D188-1 ^{32}P-labelled total DNA and isolated the non-hybridizing cosmids. All clones subsequently hybridizing with the pD188 plasmid were discarded. The remaining cosmids overlapped to form a continuous 140 kb region and were found in all virulent strains tested (D188, D188-2, NCPPB 1488, NCPPB 2551 and ATCC 12974) (Figure 2A). These sequences could not be detected in naturally avirulent isolates (NCPPB 156) or avirulent derivatives of D188 (D188-5).

A conjugative linear 200 kb plasmid encodes essential fasciation genes

The 140 kb DNA region did not correlate with any known *R.fascians* plasmid yet it could be lost by curing the wild-type strain (Desomer *et al.*, 1988). This prompted us to search for additional extrachromosomal elements in D188. CHEF pulsed field gel electrophoresis of undigested total DNA of this bacterium revealed the presence of a large DNA molecule of ~200 kb present only in virulent strains. As

Table I. Virulence phenotypes and plasmid content of different *R.fascians* strains

R.fascians strain	Resistance marker	pD188-type plasmid	Fi plasmid	Virulence	Origin
D188	Cd^R	+	+	+	A
D188-1	Cd^S	−	−	−	A
D188-2	Cd^S	−	+	+	A
D188-5	Cd^S, Str^R	−	−	−	A
D188-7	Cd^R, Str^R	+	−	−	A
D188-12	Cd^R, Str^R, Phl^R	−	−	−	B
D188-14	Cd^S, Cm^R	−	+	+	C
D188-15	Cd^S, Cm^R, Str^R	−	+	+	This work
NCPPB 1488	Cd^R	+	+	+	A
NCPPB 2551	Cd^R	+	+	+	A
ATCC 12974	Cd^R	+	+	+	A
NCPPB 156	Cd^S	+	−	−	D

A, Desomer *et al.* (1988); B, Desomer *et al.* (1991); C, Desomer *et al.* (1990); D, Laboratorium voor Microbiële Genetica, Universiteit Gent, B-9000 Gent, Belgium.

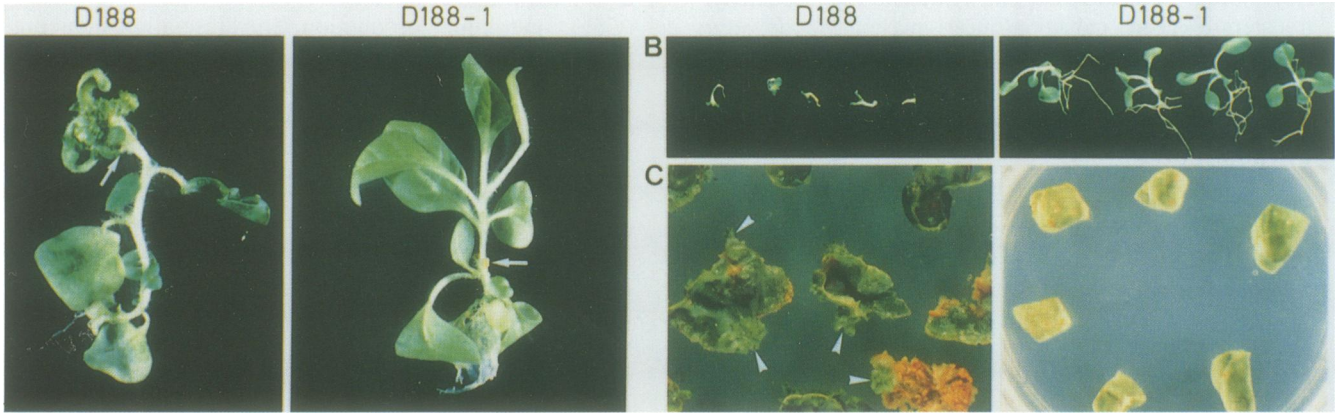

Fig. 1. Infection of *R.fascians* on *N.tabacum*. Decapitated plants (A), seeds (B) and leaf discs (C) were infected as described in Materials and methods. **A.** The leafy gall (arrow: inoculation site) was visible after one month, using the virulent strain D188 whereas no morphological alteration was observed with the strain D188-1. **B.** D188 induces stunting of the seedling and only the two cotyledons are developed. D188-1 has no inhibitory effect on plant growth. **C.** Wart-like outgrowths were observed one month after infection with D188. In contrast, D188-1 did not provoke any proliferation.

shown in Figure 2B, this 200 kb molecule hybridized with sequences from the identified 140 kb DNA virulence region (cloned in pJGV05607; panels 2 and 4), but not with chromosome-associated genes coding for pigment production (present in pJGV13602; Figure 2B, panel 4). A probe corresponding to the pD188 circular plasmid (pJGV5606) hybridized to the origin of the CHEF gels (unless lysates were restricted prior to the electrophoresis; data not shown), confirming that similarly sized circular molecules did not migrate into the gel under the conditions used. This indicated that the fasciation genes were on a, hitherto unsuspected, linear extrachromosomal element. Noteworthy is that an additional 4 megabase (Mb) DNA molecule, hybridizing with the chromosomal probe, was observed in all strains (Figure 2B, panel 4). This could indicate that the *R.fascians* chromosome is also a linear DNA molecule. Under several CHEF and PFGE conditions, these bands comigrated with 4 Mb and 200 kb standards, respectively. To verify that chromosome-sized circular molecules remain intact in these conditions, we prepared and analysed lysates from *E.coli* cells. All of this DNA was retained in the origin (data not shown).

Other *R.fascians* strains were tested and only the virulent ones contained similarly sized linear plasmids (Table I). Linear plasmid deletion mutants, detected by their hybridization pattern against the fasciation-specific cosmids (see below), contained linear molecules with higher mobility in pulsed field electrophoresis (Figure 2B, panel 3, lanes B and C). A restriction map of pFiD188 was constructed (Figure 3A) using the low frequency restriction enzymes for high GC content genomes, *Ssp*I, *Dra*I, *Ase*I and *Hpa*I. The pattern of fragments revealed by CHEF analysis and hybridization to pFiD188 (Figure 3B) or to selected subclones (summarized in Table II) confirmed the size of this molecule. The sites were also mapped, when possible, on the 140 kb cloned region.

The conjugative nature of this plasmid was tested using a strain with the chloramphenicol resistance (Cm^R) gene from pRF28 integrated into the linear plasmid by single homologous recombination (Figure 3A). This resulting virulent strain (D188-14; Table I) was conjugated with the avirulent mutant D188-5 [a streptomycin-resistant (Str^R) derivative of D188-1]. Cm^RStr^R transconjugants (D188-15) were obtained with a frequency of 10^{-4}. All now possessed the linear plasmid (Figure 2B, panel 1, lane C) and regained

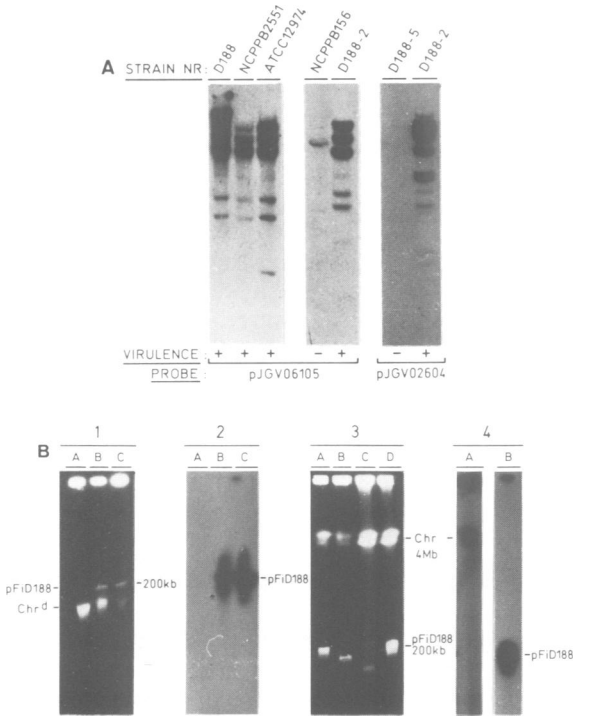

Fig. 2. A. Identification of fasciation-associated DNA. Southern hybridization of fasciation-specific cosmid probes (pJGV06105 and pJGV02604) with *Bam*HI-cleaved total DNA from virulent (NCPPB 2551, ATCC 12974, D188-2 and D188) and avirulent (NCPPB 156 and D188-5) strains. **B**. Fasciation-specific cosmids are located on a linear plasmid of 200 kb. **Panel 1**. Total DNA lysates from avirulent strain D188-5 (lane A), virulent strains D188 (lane B) and D188-15 (lane C) were separated on a CHEF gel (1% agarose in 0.5 × TBE gel, fixed angle 120°, pulse time 30 s, 6 V/cm, 12 h) and stained with EtBr. pFiD188 is the linear plasmid and Chr^d is degraded DNA. **Panel 2**. Southern hybridization of the CHEF gel in panel 1, with the ^{32}P labelled cosmid pJGV05607. **Panel 3**. CHEF gel (as in Materials and methods) containing lysates from deletion mutants of pFiD188 and stained with EtBr. Lanes A–C: M4, *fas*3 and *fas*4 (deletions given in Figure 5); lane D, D188 control. Chr, chromosomal DNA. **Panel 4**. Southern hybridization of lane D from panel 3 (D188 lysate) with lane A, the chromosomal probe pJGV13602, and lane B, the D188-2 cosmid pJGV05607. Sizes were estimated by comparison with reference lanes containing either λ multimer ladder or *Schizosaccharomyces pombe* chromosomes (BRL).

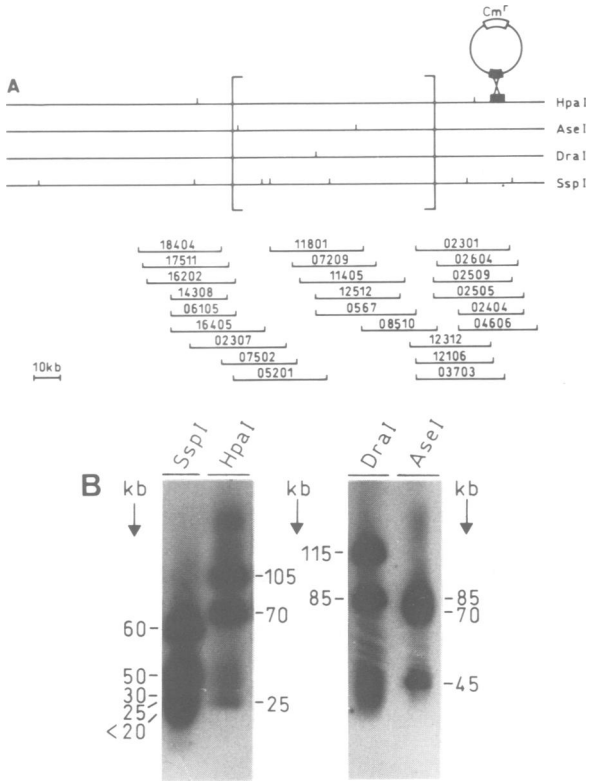

Fig. 3. Restriction map of pFiD188. **Panel A**. Macrorestriction map of the linear plasmid pFiD188 with *Hpa*I, *Ase*I, *Dra*I and *Ssp*I. The fasciation-specific cosmids were overlapped to form a contiguous 140 kb region and located on the linear plasmid by hybridization against CHEF gels. The identified phytopathogenicity region of pFiD188 (Figure 5) is shown between brackets and the introduction of the Cm^R gene via single homologous recombination is schematically shown. **Panel B**. Southern hybridization of CHEF gels containing restricted total D188 DNA (resolved between 20 and 200 kb) with ^{32}P-labelled pFiD188 DNA. DNA sizes (arrows) were determined by comparison with a λ DNA ladder.

Table II. Restriction fragment sizes (in kb) revealed by CHEF after digestions with infrequently cutting endonucleases and hybridization with pFiD188

*Dra*I	*Hpa*I	*Hpa*I−*Dra*I	*Ase*I	*Ase*I−*Dra*I	*Ase*I−*Hpa*I	*Ssp*I
115[a,b]	105[a,b]	70[a]	85[a,c]	85[a,c]	70[a]	60[c]
85[b]	70[a,c]	60[b]	70[b]	70[b]	2 × 45[a,b]	50
	25[b]	45[a]	45[a]	30[a]	25[b]	30
		25[b]		15	15[a]	25
						<20[d]

[a]Fragments revealed after hybridization with cosmid pJGV16202.
[b]Fragments revealed after hybridization with cosmid pJGV02604.
[c]Fragments revealed after hybridization with a 10 kb labelled DNA fragment located at 5 kb from the most left *Hpa*I site (orientation as in Figure 3A). These data indicate that another *Ssp*I site should be present at 10 kb from the left border of pFiD188.
[d]Additional small fragments were seen in CHEF electrophoresis (Figure 3B).

their fasciation inducing phenotype (Table I). Control conjugations between donor strains with the Cm^R gene integrated into the chromosomal pigment genes (Desomer *et al.*, 1991) and an appropriate acceptor (D188-12) yielded no transconjugants (frequency $<7 \times 10^{-9}$). Therefore, we inferred that this large linear extrachromosomal element pFiD188 (for fasciation-inducing plasmid) is conjugative.

Fasciation is a polygenic trait

D188 mutants were generated by insertion of the non-replicating construct pRF32. Integration of this plasmid is often associated with deletion of flanking DNA sequences (Desomer *et al.*, 1991). Mutants with integration events in pFiD188 were enriched by conjugation to D188-5. This step also eliminated mutations reducing replicative stability or conjugation ability of the linear plasmid. The resulting 63 different mutants on pFiD188 were screened for virulence using the pathogenicity assays described.

Virulence assays distinguished three phenotypic classes. Fourteen mutants were avirulent (class I) as judged from their inability to fasciate decapitated plants or isolated tobacco leaf discs, and by the fact that they did not inhibit the growth of *Nicotiana tabacum* seedlings (Figure 4A, row 2). These mutants could be separated into four prototrophic and ten auxotrophic strains. All the latter required methionine in axenic culture. Three mutants (class II) had an attenuated virulence phenotype. On decapitated tobacco plants only small leafy galls were found whereas seedlings infected by these strains grew to an intermediate height (Figure 4A, row 3). Small fasciations developed after four weeks (Figure 4B, left). Two mutants were hypervirulent (class III) producing 5-fold more massive leafy galls on decapitated plants (Figure 4C), although no difference was detected in the other tests.

Identification of three loci on pFiD188 involved in phytopathogenicity

The positions of the DNA rearrangements associated with altered virulence were located on pFiD188 (Figure 5). DNA sequences adjacent to pRF32 insertions (e.g. the *Stu*I fragment indicated in Figure 5) were cloned in *E.coli* and used as probes against a collection of pFiD188 specific cosmids (Figure 3). The insertion points were determined by comparison of the restriction maps of the recovered clones (from the mutant DNA) and the wild type. Deletions were delimited by Southern hybridization with labelled cosmids pJGV08510, pJGV05607 and pJGV05201 (Figure 3). Large deletions such as *fas3* and *fas4* could be directly visualized on CHEF gels (Figure 2, panel 3). These results are summarized in Figure 5.

Class I prototrophic mutants had DNA rearrangements affecting *Bam*HI fragment 1 (*fas* locus). Mutations clustered around the *Bam*HI fragment 10a were those with attenuated virulence (*att* locus). Mutant strains provoking a hypervirulent phenotype had pRF32-generated insertions/deletions in *Bam*HI fragment 6a (*hyp* locus). Mutants with no alterations of the phenotype but in the vicinity of the identified loci were also mapped (M1 to M8) and some of them were located between the *hyp* and *att* loci (e.g. M4 and M5). The rest of the mutants, also on pFiD188, were outside this region.

The avirulent prototrophic mutants *fas*1, *fas*2 and *fas*3 could be complemented to full virulence (Figure 4E, lane 4) by introducing the replicating cosmid pJGV30202 that covers both the *fas* and *att* regions (Figure 5), confirming that the observed avirulent phenotype was caused by the insertion of pRF32. However, introduction of the same replicating cosmid into the avirulent strain D188-5 (without pFiD188) failed to restore any fasciation-inducing capacity (Figure 4E, lane 3), indicating that additional genes essential for virulence are encoded by the linear plasmid.

Chromosomal avirulence mutants

Methionine-requiring strains appeared at high frequency (10 out of the 63) and all were avirulent in the seed assay and on decapitated plants. However, addition of the amino acid to the MNC medium of the leaf disc assay (see Materials and methods) was sufficient to restore the virulence phenotype of these strains (Figure 4D), suggesting that the lack of growth *in planta* was responsible for the avirulent phenotype of these strains. Interestingly, the *R.fascians* strain NCPPB 1488 is highly virulent (Table I) yet auxotrophic for arginine (data not shown), hence the mere auxotrophic condition did not necessarily lead to avirulence.

pRF32 insertions/deletions in these avirulent mutants were distributed along pFiD188. Conjugation of several mutant linear plasmids from these auxotrophs to D188-12 [a phleomycin-resistant (Phl^R) derivative of D188-5], yielded prototrophic transconjugants with fully restored virulence. These results indicate that each of these mutants harboured two mutations, one induced by pRF32 integrated into pFiD188 that did not affect virulence, and one located on the chromosome which was responsible for the methionine auxotrophy.

The fas locus contains an isopentenyl transferase gene

The DNA sequence of the 1.9 kb *Bam*HI−*Eco*RI fragment from pFiD188 (indicated in Figure 5) was compared with the mutated sequence in the avirulent strain *fas*1 and the insertion of pRF32 was located. An open reading frame (ORF *fas*1) coding for 258 amino acids was disrupted by the pRF32 insertion (Figure 6). The deduced amino acid sequence had significant homology to isopentenyltransferases from *Agrobacterium* spp. and *Pseudomonas* spp. with several conserved amino acid stretches as indicated in Figure 7. To demonstrate the isopentenyltransferase (IPT) activity of this protein, the 920 bp *Bam*HI−*Nco*I fragment containing ORF

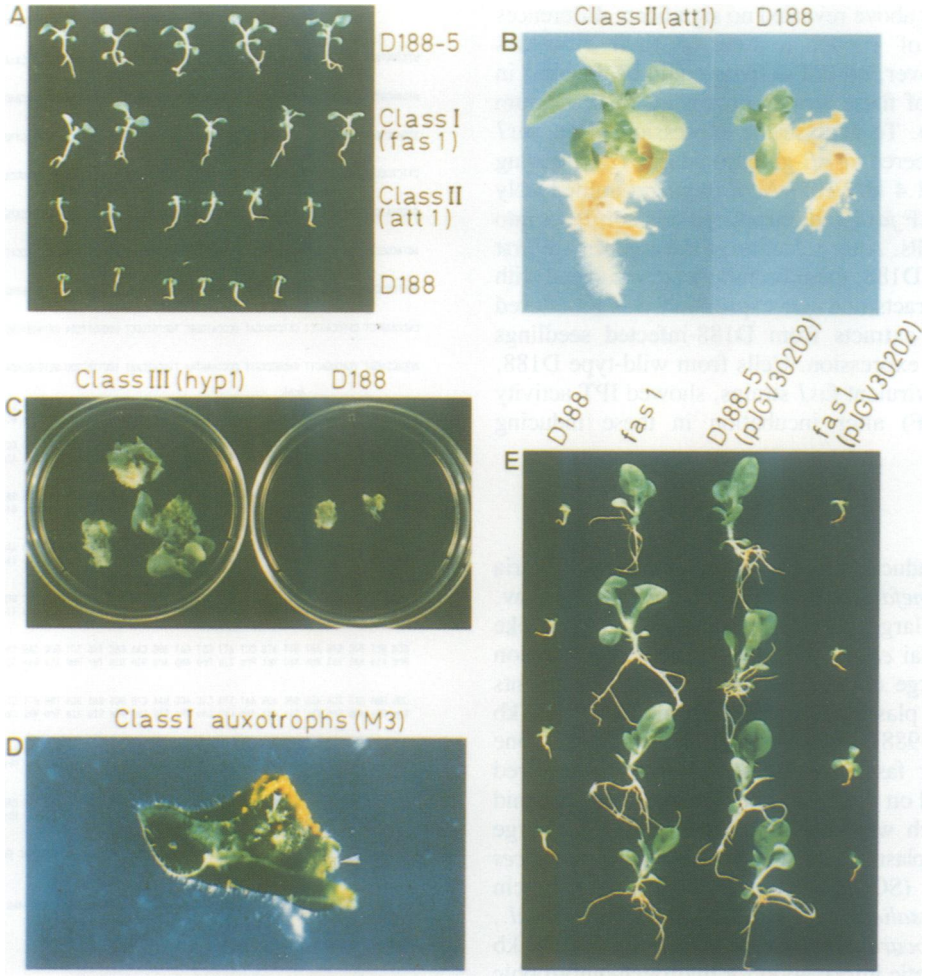

Fig. 4. Virulence phenotypes of insertional mutants in pFiD188. **Class I: avirulent**. These mutants behaved as D188-1 (Figure 1) on decapitated plants and seeds (panel A, row 2). They were separated into prototrophic (e.g. *fas*1) and auxotrophic (e.g. M3) strains. Addition of methionine at 20 μg/ml to the media for incubation of infected leaf discs, fully restored virulence (panel D) to the auxotrophs. **Class II: attenuated virulent**. Seedlings infected by these mutants (e.g. *att1*) grew to an intermediate height (panel A, row 3). After one month small fasciations were visible (panel B, left) and the main stem developed further in contrast to a D188 infected seedling (panel B, right) of the same age. **Class III: hypervirulent.** On plants, mutants (*hyp*1) form leafy galls with five times larger mass (panel C, left) than those incited by D188 in equal periods (panel C, right). **Complementation of class I mutation**. Panel E shows seedlings infected with D188 (lane 1), *fas*1 (lane 2), D188-5 harbouring the pJGV30202 cosmid (lane 3), and *fas*1 harbouring the same cosmid (lane 4).

fas1, starting from the second in-frame ATG codon located 18 nucleotides downstream from the presumed start, was cloned in pJB66 (pRIPT2) for expression in *E.coli* NFI cells. Extracts from heat-induced cells harbouring this construct were assayed for IPT activity. Fractionation of ^{3}H-labelled assay products on a reversed phase HPLC column revealed that 95% co-eluted with the internal isopentenyladenine (i^{6}Ade) standard (Figure 8B). No IPT activity was found when (i) isopentenyl pyrophosphate was omitted from the reaction mixture, (ii) the cells were not heat induced (data not shown) or (iii) extracts from NF1[pJB66] cells containing only the vector were assayed (Figure 8A). Culture supernatants of *E.coli* strains NF1[pJB66] and NF1[pRIPT2] were screened with anti-i^{6}Ade and anti-io^{6}-Ade (anti-zeatin) antibodies and significant secretion of cytokinins was found only when the expression of the chimeric *ipt* gene was induced by a heat shock (Table III).

The ipt gene is specifically expressed during the interaction of R.fascians with plants

Examination of culture supernatants of virulent wild-type D188 and avirulent *fas1 R.fascians* strains by the same

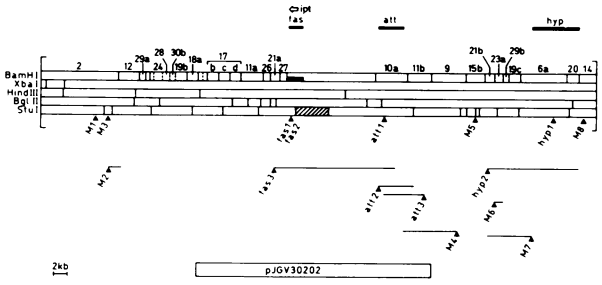

Fig. 5. Phytopathogenicity loci of pFiD188. The set of overlapping cosmids given in Figure 3A was used to establish a restriction map for *Hind*III, *Bam*HI, *Bgl*II, *Xba*I and *Stu*I of the bracketed region of pFiD188. M1 to M8 are mutations in pFiD188 that did not alter virulence of the harbouring strains. *fas*, *att* and *hyp* are class I, class II and class III mutants, respectively; *fas4* is a deletion of this whole region and is therefore not indicated. Filled arrow heads, insertions; lines, deleted regions. The empty bar is the replicating cosmid pJGV30202 complementing the *fas1* mutation. A striped bar indicates the *Stu*I fragment, adjacent to the insertion point of *fas*1, used for isolation of pJGV30202; a half-filled bar is the 1.9 kb sequenced DNA fragment (Figure 6) from the *fas* locus. Filled black bars indicate the identified loci. The open arrow indicates the ORF coding for isopentenyltransferase.

assays as described above revealed no significant differences in the amounts of i^6Ade, io^6Ade or their ribosides (Table III). Moreover, no IPT activity could be detected in cell-free extracts of these strains grown in YEB medium (Figure 8C and E). To analyse the expression of the *fas1* locus, we engineered a β-glucuronidase (*gus*)-coding sequence with the 1.4 kb *Nco*I−*Nco*I fragment immediately upstream from ORF *fas1* and introduced this construct into wild-type D188 cells. After selection of the appropriate first recombinant in pFiD188, these bacteria were incubated with different plant extracts and *gus* expression was monitored (Table IV). Only extracts from D188-infected seedlings could activate *gus* expression. Cells from wild-type D188, but not from the avirulent *fas1* strains, showed IPT activity (Figure 8D and F) after incubation in these inducing conditions.

Discussion

The hyperplasia-inducing genes of Gram-negative bacteria *Agrobacterium tumefaciens* and *Pseudomonas syringae* pv. *savastanoi* lie on large, circular plasmids (Van Larebeke *et al.*, 1975; Comai *et al.*, 1982). A variety of extraction procedures for large circular extrachromosomal elements revealed several plasmids ranging from 70 to 180 kb (Desomer *et al.*, 1988) in virulent *R.fascians* strains. None were essential for fasciation. Instead, virulence-required genes were located on a 200 kb, conjugative, linear plasmid (Figure 2B), which we have designated pFiD188. Large conjugative linear plasmids have been found in *Streptomyces coelicolor* A3(2) (SCP1; involved in methylenomycin biosynthesis), *S.lasaliensis* (pKSL, 520 kb; Kinashi *et al.*, 1987) and in *Nocardia opaca* (e.g. pHG201, 270 kb containing the genetic information for hydrogen-autotrophic growth; Kalkus *et al.*, 1990). The conjugative ability of these plasmids allows their dissemination amongst actinomycetes and related organisms. Correspondingly, pFiD188-like plasmids were identified in all virulent strains of *R.fascians* (Figure 2A; Table I). In addition, several smaller linear plasmids were identified in different eukaryotic organisms (reviewed by Meinhardt *et al.*, 1990), bacteria (reviewed by Sakaguchi, 1990) and in the spirochaete genus *Borellia* (Barbour and Garon, 1987). The latter parasites are the only prokaryotic organisms hitherto shown to possess a linear chromosome (Ferdows and Barbour, 1989). Our studies have shown that *R.fascians* might be the first free-living bacterium also to have a linear chromosome (Figure 2B). We think it is unlikely that the 4 Mb DNA was derived from a circular chromosome that had been sheared during the lysis treatment because (i) the DNA bands were discrete and always of the same size when different CHEF and PFGE conditions were used, and (ii) large circular chromosomes, such as those present in *E.coli*, did not migrate in any of these conditions. Further studies on the ends of these linear molecules should help to elucidate this issue.

Insertion mutagenesis of pFiD188 has detected three loci that were necessary for normal fasciation. Inactivation of one (*att*) reduced virulence. This gene might therefore be important for attachment or growth on the host cells, produce auxiliary chemicals that facilitate tumour formation, or enhance expression of the primary tumour-inducing genes. Inactivation of at least one gene in the *hyp* locus leads to the opposite phenotype so that tumours grow more rapidly.

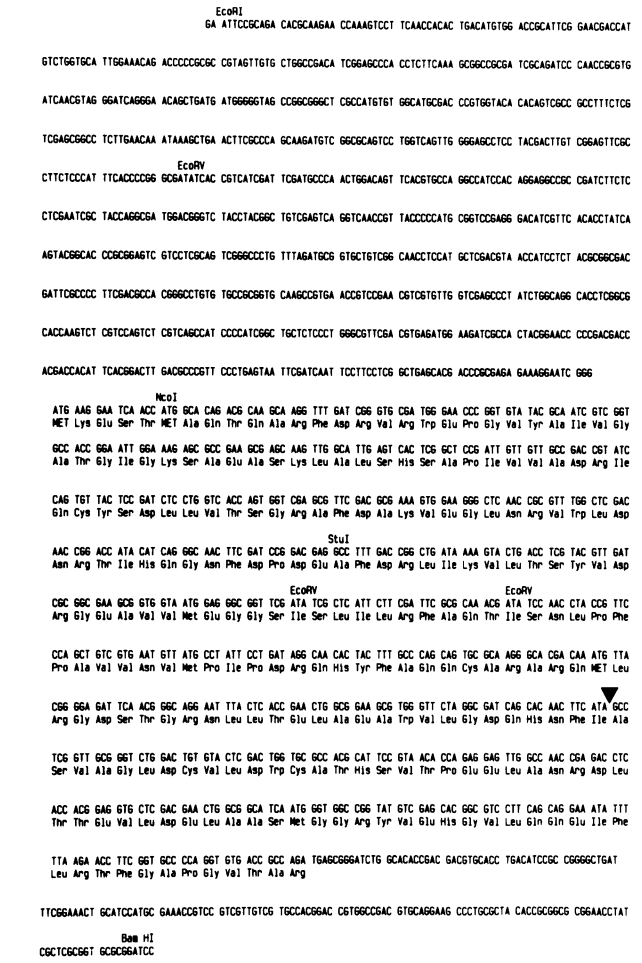

Fig. 6. Nucleotide sequence of the 1.9 kb *Bam*HI−*Eco*RI fragment (of *Bam*HI fragment 1) corresponding to locus *fas* (EMBL accession number X62428). The insertion point in mutant *fas1* is indicated by a triangle and the deduced amino acid sequence of the open reading frame encoding isopentenyltransferase is shown.

Thus this gene, like the *Pseudomonas iaaL* gene (Romano *et al.*, 1991), might modify plant hormones to a less active form, or repress expression of other virulence functions. The methionine-requiring *R.fascians* strains are chromosomal avirulent mutants that appear frequently during the mutagenesis and/or conjugation procedures, suggesting that the methionine locus is preferentially inactivated by a DNA rearrangement or modification. This amino acid requirement is particularly common among rhizosphere bacteria and restricts the growth of these prokaryotes *in planta* (Hedges and Messens, 1990). The *R.fascians* mutants may be capable of obtaining nutrients from the plant except at least methionine since other auxotrophs (Table I) are virulent. Supplementation of methionine restores pathogenicity to these mutants (Figure 4D) either by sustaining basic bacterial metabolism or by fulfilling some function related to the interaction with the host.

What is more clear is that the ORF interrupted by pRF32 insertion in the *fas* locus codes for a protein homologous to the isopentenyltransferase gene products from the well characterized hyperplasia or root-inducing Gram-negative bacteria *A.tumefaciens*, *A.rhizogenes*, *Pseudomonas savastanoi* and the pathogenic *P.solanacearum* (Heidekamp *et al.*, 1983; Goldberg *et al.*, 1984; Beaty *et al.*, 1984;

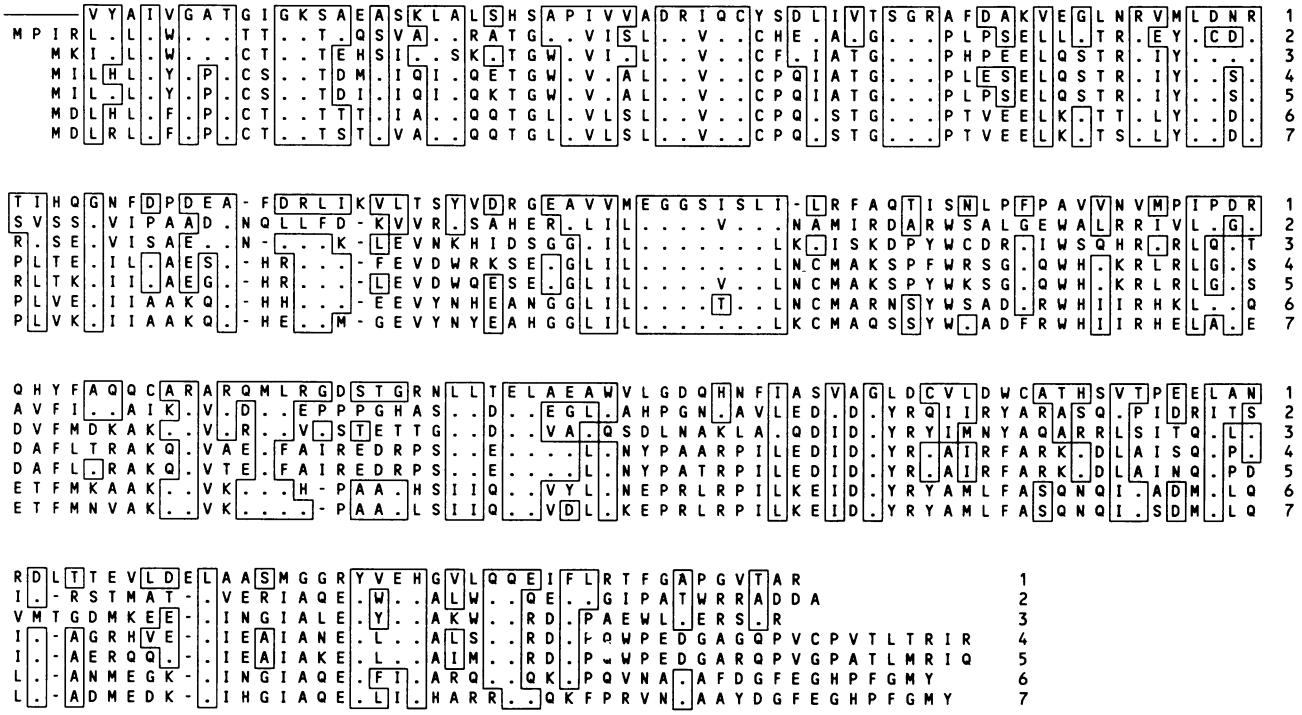

Fig. 7. Comparison of the deduced amino acid sequences of genes *tzs* (line 2; Akiyoshi *et al.*, 1989) and *ptz* (line 3; Powell and Morris, 1986) of *Pseudomonas* spp., the *tzs* gene of *Agrobacterium tumefaciens* (line 4; Beaty *et al.*, 1984) and *Agrobacterium rhizogenes* (line 5; Regier *et al.*, 1989), the *tmr* gene from *A. tumefaciens* octopine (line 6; Heidekamp *et al.*, 1983) and nopaline (line 7; Goldberg *et al.*, 1984) strains, and ORF *fas1* (line 1) of *R.fascians*. Identical (points) or structurally similar (boxes) amino acids between ORF *fas*1 and either of the isopentenyltransferases are indicated. Gaps (dashes) are introduced to maximize the alignment. ORF *fas*1 has a potential 21 amino acid amino-terminal extension, compared to the other proteins, indicated on the figure by a solid line.

Powell and Morris, 1986; Akiyoshi *et al.*, 1989; Regier *et al.*, 1989) (Figure 7). The low overall homology (20 to 26% at amino acid level) may indicate that this gene has not been acquired recently from a Gram-negative plant pathogen. The conservation of the amino-terminal part of the proteins, emphasized up to now (Akiyoshi *et al.*, 1985; Powell and Morris, 1986), is weakened by our result although this could equally reflect different enzymatic specificities. The conserved peptide (amino acid positions 111−123) could be involved in building up a functional domain important for enzymatic activity. The 21 amino acid terminal extension only present in the *R.fascians* IPT polypeptide could be needed for the processing of this protein although it bears no resemblance to other known signal sequences.

The disease symptoms developed after *R.fascians* infection (inhibition of root formation, teratoma and abundant shoot induction) are similar to the aberrant morphologies associated with cytokinin overproduction in plants ('shooty' tumours, Leemans *et al.*, 1982) and early reports (Balázs and Sziráki, 1974) showed a considerably more extractable cytokinin activity from *R.fascians*-infected *Pelargonium zonale* plants than from healthy ones. Low amounts of i^6Ade (24 ng/ml in kinetin equivalents) have been reported in *R.fascians* MW2 (highly virulent) culture medium supernatant (Murai *et al.*, 1980), although no immunodetectable zeatin could be found in supernatants of two out of three highly virulent strains (Akiyoshi *et al.*, 1987). Only low amounts (about 2 ng/ml) were detected in the third one. We showed that the amounts of i^6Ade and its riboside secreted by the wild-type and the avirulent *ipt*-deficient strain were the same (Table III), indicating that the *ipt* gene, which is necessary for fasciation, must be expressed specifically during interaction with the plants. Indeed, induction of *ipt* expression occurred when bacteria were incubated with extracts from D188-infected sunflower seedlings (which have phenotypes analogous to that of *N.tabacum* seedlings). The inducing compound has not been detected in extracts from normal plants (Table IV) and is currently being characterized. These signal molecules could be produced in sufficient amounts either through the action of plant genes expressed in these differentiating cells or through *R.fascians* genes involved in the biosynthesis of the active inducer from a plant precursor. The latter case covers the possibility that the biosynthetic genes for these compounds are bacterially encoded and transferred to plant cells as it has been shown for opine synthesis in *Agrobacterium* spp. (Zambryski, 1988).

Interestingly, high amounts of *trans*-zeatin and isopentenyladenine have been demonstrated in several *Pseudomonas savastanoi* strains (e.g. 400 ng/ml for EW1006) and in *A.tumefaciens* C58 (5 ng/ml but a 100-fold induction occurs by addition of acetosyringone; Akiyoshi *et al.*, 1987; Powell *et al.*, 1988). These bacteria are unable to induce the development of multiple shoots out of the site of infection. Moreover, *P.savastanoi* mutants, which no longer produce indole-3-acetic acid, have poor gall-forming ability (Surico *et al.*, 1984; MacDonald *et al.*, 1986) and do not produce shoots. These data indicate that fasciation is a complex phenotype and is not only dependent on cytokinin excretion. A cosmid spanning *fas* and *att* cannot restore the virulence of a linear plasmid-deficient strain (despite its higher copy number compared with pFiD188) (Figure 4) demonstrating that at least one additional essential gene is located on the extreme left or right arms of pFiD188.

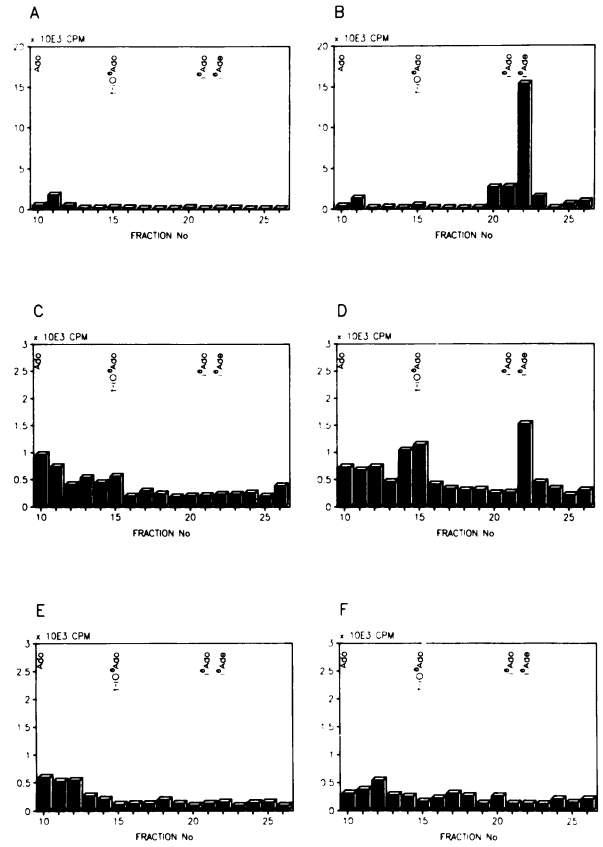

Fig. 8. Expression of ORF *fas*1 shows IPT activity. Radioactivity profile of IPT assay products separated on HPLC using extracts from: **A**, *E.coli* NF1 harbouring the pJB66 vector; **B**, *E.coli* NF1 harbouring the ORF *fas1* expression construction pRIPT2; **C**, D188 cells; **D**, D188 cells induced with a fasciated tissue extract; **E**, *fas1* cells; **F**, *fas1* cells induced as D. Elution time of cytokinin standards are shown. Ado, adenosine; t-io^{6}Ade, *trans*-zeatin; i^{6}Ado, isopentenyl adenosine; i^{6}Ade, isopentenyladenine.

Hypothetical functions for these other genes could be production of a different biologically active molecule that modulates the plant response to the hormone or mediation of specific transport of the hormone into plant cells.

The new features acquired by *R.fascians* to induce plant tumours suggest that this bacterium has evolved independently from the other hyperplasia-inducing bacteria to achieve a similar ecological niche. Further studies of these plant/bacterium interaction will possibly uncover novel insights into cytokinin action and shoot development in plants.

Materials and methods

Bacterial strains and plasmids

R.fascians D188 is a highly virulent strain; D188-1 and D188-2 are cured D188 derivatives, and D188-5 is a Str^R mutant derived from D188-1 (Desomer *et al.*, 1988). NCPPB 1488, NCPPB 2551 and ATCC 12974 are virulent *R.fascians* strains whereas NCPPB 156 is a natural avirulent isolate. Conjugation between *R.fascians* strains, growth conditions and selective concentrations of antibiotics have been described (Desomer *et al.*, 1988). L-methionine or arginine (20 μg/ml) was added to minimal A medium (Vervliet *et al.*, 1975) for auxotroph characterization. The JM medium used for induction experiments consists of Gamborg's B5 medium (Flow Laboratories) supplemented with 0.5 g/l MES, pH 5.5.

The construction of D188-14 has been described elsewhere (Desomer *et al.*, 1990). Briefly, pRF40 (a pRF32 derivative which contains a 4 kb insert homologous to pFiD188 and a Cm^R gene from *R.fascians*) was electroporated in D188 cells. Cm^R transformants were isolated and demonstrated (by Southern analysis) to be single homologous recombinants. D188-15 is a Str^{R}Cm^R transconjugant strain, resulting from a D188-14 × D188-5 conjugation experiment and was obtained by similar mating conditions to those used for conjugative transfer of pD188-type plasmids (Desomer *et al.*, 1988). D188-12 is a Phl^R derivative of D188-5 (Desomer *et al.*, 1991).

Escherichia coli host strains were either MC1061 (Casadaban and Cohen, 1980) or DH5α (Hanahan, 1983). Construction of the isopentenyl transferase-overexpressing plasmid was done in MC1061 (λ) and overexpression was achieved in NF1 (Van Kaer *et al.*, 1987).

pJB66 is an expression vector for *E.coli* driven by the λ promoter (Botterman and Zabeau, 1987). pRF32 is a cointegrate plasmid of pUC13 and pRF28 (a Cm^R endogenous *R.fascians* plasmid), that does not replicate in *R.fascians* (Desomer *et al.*, 1990). pJGV30202 is a cosmid selected from a partial *Sau*3A-generated library of D188 total DNA in the bifunctional cosmid vector pJGV9 (Desomer *et al.*, 1991). pD188 is a large ccc plasmid encoding Cd^R (Desomer *et al.*, 1988). Fasciation-specific cosmids were isolated by hybridization of a genomic library of *R.fascians* D188 (Desomer *et al.*, 1988) with ^{32}P-labelled D188-1 DNA. After discarding cosmids belonging to pD188, the remaining non-hybridizing ones were ordered in a continuous array by restriction fragment overlapping and cross-hybridization.

Virulence assays

Virulence of *R.fascians* strains was assayed using *N.tabacum* as host. (i) Inoculation at the wounding site of decapitated *N.tabacum* W38 plants allows symptoms to be scored after one month. (ii) Sterile tobacco W38 seeds were plated on MS medium (Murashige and Skoog, 1962) supplemented with 1% sucrose and 0.001% thiamine. Two days later, seedlings were immersed on a saturated *R.fascians* culture for 2 min, dipped on Whatman 3MM paper and transferred to fresh medium. After 15 days the virulence phenotype of the bacteria could be monitored. (iii) Leaf discs prepared from SR1 tobacco plants were infected similarly and put onto A3 medium (De Block *et al.*, 1987) with naphthalene acetic acid (NAA) (0.2 mg/l). The discs were transferred weekly to MS medium with NAA (0.2 mg/l) and triacillin (500 μg/ml) (MNC medium). Wart-like growths developed in the first month and leafy galls were obtained after two months.

Table III. Cytokinin levels in different culture supernatants from *E.coli* and *R.fascians* strains (pmol/ml medium) determined by radioimmuno assays

Strain	i^{6}Ade/i^{6}Ado[a]	io^{6}Ade/io^{6}Ado
E.coli Nf1(pRIPT2)[b]	7140/133	500
Nf1(pJB66)[b]	15/5	0.2
R.fascians D188[c]	53/10	0.2
fas1[c]	54/10	0.7

[a]The antibodies showed 10-fold less affinity for the free bases than for the ribosides; therefore both values refer to calibration curves made by free bases or ribosides, respectively.
[b]After the heat shock treatment (see Materials and methods) using M9 medium (Maniatis *et al.*, 1982).
[c]Accumulation of cytokinins during 5 days in culture on the medium described by Klämbt *et al.* (1966).

Table IV. β-glucuronidase activities of the *fas1*−*gus* gene fusions in D188 cells induced with plant tissue extracts

	GUS activity (μmol MU/h/mg protein)
D188::*fas1*−*gus*	34.7
D188::*fas1*−*gus* + extract from normal cells	33.7
D188::*fas1*−*gus* + extract from fasciated tissue	326.0

Extracts were prepared from infected or non-infected *Helianthus annuus* L.

Pulsed field gradient electrophoresis of bacterial lysates

R.fascians mid-log cultures (2 days) were centrifuged and washed in TE buffer (50 mM Tris−HCl, 20 mM EDTA, pH 8). Bacterial pellets (from 4 ml of culture) were incubated in 1 ml of TE containing lysozyme (1 mg/ml) and 20% polyethylene glycol 6000 for 4−6 h and resuspended in the same volume of 0.7% low melting point agarose, 125 mM EDTA to prepare agarose plugs. These plugs were incubated for 14 h in ES buffer (125 mM EDTA, 1% sarkosyl) with 1 mg/ml proteinase K at 50°C. After several washings with ES buffer, they were loaded on a 0.25 × TBE buffer (40 mM Tris−borate, 1 mM EDTA, pH 8) 1% agarose gel and run in contour-clamped homogeneous electric field (CHEF) (Chu *et al.*, 1986) at 220 V with a 20 s pulse and a linear gradient of the angle between 120° and 95° for 24 h (Rotaphor) unless mentioned otherwise. Afterwards, the gel was stained with 0.2% ethidium bromide. For Southern blottings, DNA was depurinated and treated as recommended for nylon membranes (Amersham). Linear plasmid DNA was purified from CHEF gels using Gene clean II kit (Bio101 Inc., La Jolla CA).

Construction of the pFiD188 restriction map

Macrorestriction mapping of the linear plasmid was carried out by digesting total DNA plugs (after several washings with TE buffer supplemented with 1 mM phenylmethylsulphonyl fluoride and equilibration in restriction buffer) with *Hpa*I, *Dra*I, *Ase*I and *Ssp*I either alone or in pairwise combination. Digested samples were resolved in pulsed field conditions for high resolution of fragments between 20 and 200 kb (1% agarose gel, 120 V, linear gradient of the pulse between 3 and 14 s, logarithmic gradient of the angle from 120° to 95° for 96 h). Southern blots of such gels were probed against pFiD188 DNA and selected subclones to determine adjacent fragments. In parallel, restriction mapping was done in the region covered by fasciation-specific cosmids by conventional techniques with a resolution of 200 bp. Only one *Ssp*I site could not be located in this way (outside this cloned region) and its position of 10 kb from the left end of pFiD188 was inferred from the difference in sizes between a *Hpa*I fragment (70 kb) and an *Ssp*I fragment revealed by the same probe (Table II, item c).

Isolation of insertion mutants on the linear plasmid pFiD188

To isolate tagged insertion mutants in *R.fascians*, we proceeded as described (Desomer *et al.*, 1991) using pRF32 but including a conjugation step to enrich for insertion mutations in the linear plasmid. After electrotransformation of D188 with 1 μg of pRF32 DNA per 500 μl electro-competent cells in 20 parallel experiments, Cm^R transformants from 40 individual plates were collected (~ 10^3 clones/plate) and conjugated in pools to D188-5 avirulent bacteria. Ten Str^R Cm^R transconjugants from each conjugation event were analysed by Southern hybridization of total DNA (*Bam*HI digested) against ^{32}P-labelled pRF32 plasmid DNA. Sixty-three mutants differing in their hybridization pattern were selected and individually tested for virulence towards *N.tabacum* plants. The stability of these mutations was assessed by comparison of the hybridization patterns with ^{32}P-labelled pRF32 of total DNAs from mutant strains before inoculation and after reisolation of bacteria from infected plants.

Localization of pRF32 insertion mutants and cloning of the tagged genes

DNA rearrangements (insertions/deletions) in mutants were analysed by preparation of total DNA and cloning of the *R.fascians* sequences adjacent to the insertion point in *E.coli* as described (Desomer *et al.*, 1991). Fragments containing *R.fascians* sequences were labelled and either used for screening the D188 cosmid library or directly hybridized against the pFiD188 cosmids. To locate the insertion point, restriction maps of the recovered clones were compared with their cloned wild type counterparts. Nucleotide sequencing of the insertion borders and target fragments permitted localization of the insertion at the nucleotide level. Deletions associated with pRF32 insertions were delimited by hybridization of total DNA from the mutants against labelled pFiD188 cosmids.

The region of the linear plasmid which carried the identified loci was mapped (*Bam*HI, *Hin*dIII, *Stu*I, *Xba*I and *Bgl*II) in more detail using the different cosmids covering the region. Final map positions were confirmed using subcloning, cross-hybridizations of purified fragments and double digestion with other enzymes. *Bam*HI restriction fragments were named by descending molecular weight in a larger region than the one depicted here but original nomenclature was kept.

Complementation of class I mutants

The replicating cosmid pJGV30202, containing both *fas* and *att* loci, was introduced by electroporation in the *fas*1, *fas*2, and *fas*3 mutant cells. Phl^RCm^R colonies were isolated and the presence of the plasmid was confirmed. In addition, Southern hybridizations with pJGV30202 revealed both wild-type and mutant fragments in total DNA from these transformants. They were tested for virulence on *N.tabacum*. The cosmid was also introduced in D188-5, selecting for Str^RPhl^R and analysed similarly.

Construction of an E.coli strain expressing the isopentenyltransferase gene

A 920 bp *Bam*HI−*Nco*I fragment (starting from the methionine located 5 amino acids behind the proposed start codon of the ORF encoding *ipt* and including 3′-untranslated sequences) was cloned in pJB66 (pRIPT2) under control of the P_r promoter of phage λ using *E.coli* MC1061λ as the host bacterium (Botterman and Zabeau, 1987). This plasmid was used to transform *E.coli* NF1 carrying the thermosensitive repressor (cI857) of phage λ. NF1(pRIPT2) cells were grown at 28°C in LB to an $OD_{660\ nm}$ of 0.4, heat-shocked for 20 min at 42°C and further incubated for 3 h at 37°C. Cells were harvested by centrifugation, washed twice in TE buffer (50 mM Tris−HCl, 10 mM EDTA, pH 8) and concentrated 20-fold in buffer A (10 mM Tris−HCl, 10 mM magnesium acetate, 6 mM KCl, 6 mM β-mercaptoethanol, pH 7.5). Lysis was accomplished with a sonicator cell disrupter B-10 (Branson Sonic Power Corp.) in three 20 s bursts at 40 W output. The cell debris (pelleted by centrifugation at 12 000 *g* for 15 min) was discarded and glycerol was added to the supernatant (20% v/v final concentration) for storage at −20°C. Protein concentration was determined according to Bradford (1976).

Isopentenyl transferase activity and cytokinin assays

IPT activity was assayed in a final volume of 400 μl essentially as described (Barry *et al.*, 1984) using 1 μCi of [^{3}H]AMP (Amersham), 1 mM of isopentenyl pyrophosphate (Cornforth and Popják, 1969) and 100 μl of the above described sonicate (typical protein concentration 2 mg/ml). For *R.fascians*, sonicates were obtained by four 10 s bursts at 40 W. Reaction mixtures were treated with alkaline phosphatase (1 h at 37°C at pH 9.5), diluted in 0.1% (v/v) trifluoroacetic acid (TFA) and passed through a C18 cartridge (SEP-PAK Waters Associates). Retained material was eluted with methanol, dried *in vacuo* and dissolved in 0.1% aqueous TFA (buffer A). Analysis was done by reversed phase chromatography on a C18 Lichroma Vydac 300 Å analytical column using the following elution gradient: 30 s at 0% buffer B, 30 min linear gradient from 0 to 25% buffer B, 4 min linear gradient from 25% to 100% buffer B, and 11 min at 100% buffer B (buffer B: 70% acetonitrile, 0.1% aqueous TFA). One millilitre samples were collected and dissolved in 3.3 ml of liquid scintillation cocktail (ReadySafe, Beckman) to measure incorporated radioactivity.

Cytokinin levels accumulated in supernatants of bacterial cultures were determined by radioimmuno assay (Akiyoshi *et al.*, 1987) of total samples (M9 medium for *E.coli*; Maniatis *et al.*, 1982; and asparagine medium for *R.fascians* strains; Klämbt *et al.*, 1966).

Induction assays

R.fascians D188::*fas1*−*gus* cells were resuspended in JM medium ($OD_{660\ nm}$ = 0.2) and incubated for 12 h in the presence of plant tissue extracts (1:1000 v/v). Cell-free extracts were prepared as mentioned above in 50 mM $NaPO_4$ (pH 7.0), 1 mM EDTA, 10 mM β-mercaptoethanol. GUS activity was measured (Jefferson *et al.*, 1986) using 4-methyl-umbelliferyl-β-D-glucuronide. Fasciated or control tissue extracts were prepared by maceration (1 g fresh weight/ml) in 2 mM MES (pH 5.6). After centrifugation at 12 000 *g* for 10 min, the supernatant was filtered (0.22 μm; Millipore) and stored at −20°C.

General cloning and analysis techniques

For general cloning pUC18 (Yanisch-Perron *et al.*, 1985) was used. Plasmid DNA preparations from *E.coli*, restriction enzyme digestions, ligations and Southern hybridizations were performed using standard conditions (Maniatis *et al.*, 1982). Nucleotide sequences were determined using the T7 sequencing kit from Pharmacia (Uppsala, Sweden). Sequence analysis was performed using the programs of the Intelligenetics Suite and databases were screened by FASTDB Software (Brutlag *et al.*, 1990).

Plasmid, total DNA isolations, and electroporation conditions of *R.fascians* have been reported (Desomer *et al.*, 1990).

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